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Antiviral and Immunostimulant Activities of *Andrographis paniculata*

CHURIYAH[1*], OLIVIA BUNGA PONGTULURAN[1], ELRADE ROFAANI[1], TARWADI[2]

[1]Center of Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology, Building 610 LAPTIAB, Puspiptek Area, Serpong–Tangerang 15314, Indonesia
[2]Biotech Center, Agency for the Assessment and Application of Technology, Building 630, Puspiptek Area, Serpong–Tangerang 15314, Indonesia

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***Andrographis paniculata* (Burm. f.) Nees is a medicinal plant which was reported to have anti HIV, anti pathogenic bacteria and immunoregulatory activities. The research purpose was to investigate the activity of *Andrographis paniculata* ethanol extract as antiviral and immunostimulant. *A. paniculata* leaves oven-dried, then grinded and macerated with ethanol 90%, and the extract then analyzed using High Performance Liquid Chromatography (HPLC) to determine the content of active compounds andrographolide. The antiviral activity of the extract was determined by observing its ability on inhibiting virus load in A549 cells transfected with Simian Retro Virus (SRV) by Real Time – Polymerase Chain Reaction (RT-PCR) analysis. The immunostimulant activity of extract was determined by its ability to induce lymphocytes cell proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Our result indicated that the *A. paniculata* ethanol extract inhibited the SRV virus titer similar to the positive control Lamivudine, and it was not toxic to the A459 cell line. Furthermore, low concentration (1 µg/mL) of *A. paniculata* extract could stimulated lymphocyte cell proliferation about 38% compared to the control lymphocyte cell without any treatment.**

Keywords: *Andrographis paniculata*, anti-viral, immunostimulant

INTRODUCTION

Andrographis paniculata (Burm. f.) Nees (Acanthaceae) is a medicinal plant used in many countries. Major constituents of *A. paniculata* are diterpenoids, flavonoids and polyphenols. Among the single compounds extracted from *A. paniculata*, andrographolide is the major compound in terms of bioactive properties and abundance.

Methanolic extracts of *A. paniculata* as well as chloroform and hexane extracts were reported to inhibit the growth of bacterial and fungal pathogens (Bobbarala *et al*. 2009). The aqueous extract of *A. paniculata* against anti-HIV was ruled out by testing the inhibitory activities against HIV in the H9 cell line (Chao & Lin 2010). A phase I dose-escalating clinical trial of andrographolide in HIV positive patients reported a significant rise in the mean $CD4^+$ lymphocyte level of HIV patients. Andrographolide inhibited HIV-induced cell cycle disregulation, leading to a rise in $CD4^+$ lymphocyte levels in HIV-1 infected individuals (Calabrase *et al*. 2000). Andrographolide, neoandrographolide and 14-deoxy-11,12-didehydroandrographolide demonstrated viricidal activity against herpes simplex virus 1 (HSV-1) (Wiart *et al.* 2005). The *A. paniculata* ethanol extract and andrographolide inhibit the expression of Epstein-Barr virus (EBV) (Lin *et al*. 2008). A recent *in vitro* study investigated the anti-influenza activity of *A. paniculata* in canine kidney cell line infected with H1N1, H9N2, or H5N1 (Chen *et al*. 2009). Andrographolide and their derivatives displayed anti-HIV activity *in vitro* by inhibiting gp120-mediated cell fusion of HL2/3 cells (expressing gp120 on its surface) with TZM-bl cells (expressing CD4 and co-receptors CCR5 & CXCR4) (Uttekar *et al*. 2012).

Many of andrographolide analogues indicating immunostimulatory properties (Chao & Lin 2010). Andrographolide was reported to have immunoregulatory activities with different effects in different immune disease models, playing a role as a modulator of altered immune responses. In tumor-bearing mice it enhanced natural killer cell activity, increased secretion of IL-2 and IFN-γ by T cells and thereby inhibited the tumor growth. However, in autoimmune encephalomyelitis mice, it interfered maturation of dendritic cells, induced antigen-specific tolerance and thus prevented detrimental autoimmune responses (Wang *et al*. 2010). *In vivo* study using

*Corresponding author. Phone/Fax: +62-21-7560536,
E-mail: churiyah_zafrollah@yahoo.com

mice model have shown that *A. paniculata* was a potent stimulator of immune system in two ways, namely the antigen specific response that antibodies was made to counteracted invading microbes, and non specific immune response that macrophage cell scavenged and destroyed invader (Kumar *et al*. 2011).

The aim of our research was to investigate the activity of the ethanol extract of *A. paniculata* as antiviral and immunostimulator. The activity of extract as immunostimulant was determined on inducing lymphocytes cell proliferation using MTT assay, while as antiviral was observing its ability on inhibiting SRvirus titer in A549 cells transfected with SRV by RT-PCR.

MATERIALS AND METHODS

Preparation of Ethanol Extracts of *A. paniculata.* Simplicia *A. paniculata* leaves were oven-dried, then grinded with a grinder until becomes a fine powder. Furthermore, the powders was extracted by maceration with ethanol 90% and stirred for 24 h. The macerate then filtered with a vacuum pump and evaporated with a rotary evaporator at 40 °C to obtain a viscous extract. The ethanol extract then analyzed using High Performance Liquid Chromatography (HPLC) to determine the content of active compounds andrographolide. About ten milligrams of extract was dissolved in 10 mL of methanol and then filtered with a 0.45 µm pore size of 13 mm diameter membrane. The HPLC was setting by the linear gradient system from 70:30 to 100:0 then 70:30 with methanol:distillate water eluent, with a flow rate of 1 mL/min for 20 min and analyzed at the wavelength of 254 nm.

Isolation and Proliferation of Peripheral Blood Lymphocyte. Blood samples were taken aseptically from healthy donors and stored in sterile tubes which already contained 0.1% EDTA as an anticoagulant. Blood samples then aseptically transferred into a sterile centrifuge tube and centrifuged for 5 min at 214 g. Heavier part of the blood (red blood cells) will be at the bottom and the plasma will be on top.

The plasma was removed and placed into the new sterile conical tube then diluted with 50% Roswell Park Memorial Institute (RPMI) 1640 basal medium, mixed gently, then layered onto Ficoll histopaque. After centrifuged at 594 g for 30 min, the white range buffy coat collected into the new conical tube contained the basal medium, and centrifuged at 95 g for 50 min, then washed twice with the basal medium. The lymphocyte cells were resuspended in RPMI 1640 medium with 10% heat inactivated fetal bovine serum (FBS) and 1% Pen-Strep antibiotic (100 Units/mL Penicillin and 100 µg/mL Streptomycin).

Proliferation of lymphocyte cells was measured using MTT method. The yellow color of MTT changed to purple by mitochondrial succinate dehydrogenase of living cells. Briefly, 5×10^5 cells per well in a 96-well plate were stimulated with different concentration of extract (Table 1 for the details of extract concentration) and mitogen as positive control which were 5 µg/mL of Phytohaemagglutinin (PHA) and Concanavaline A (Con A). Control wells received only medium without extract or mitogen addition. After 72 h of culturing at 37 °C in a humidified incubator with 5% CO_2, 10 µL of 5 mg/mL MTT was added to each well. After incubation at 37 °C with 5% CO_2 for 4 h, 100 µL of 10% SDS was added and mixed to release the purple colour from the cells. Optical density at 570 nm was measured using microplate reader (Arokiyaraj *et al*. 2007)

Cytotoxicity Assay. Continuous A549 cells were grown in the culture medium containing DMEM medium with 10% FBS, and 1% Pen-Strep antibiotics, and incubated at 37 °C in a humidified incubator with 5% CO_2. Confluent A549 cell was tripsinized and seeded at 5×10^4 cells/well in 96-well microplates, incubated for 24 h at 37 °C to allowed cell attached onto the plate. Then the culture medium was discarded and fresh medium culture contained different concentrations of the extract were added and incubated for 24 h. The 50% inhibitory concentration (IC_{50}) was defined by the MTT method. Briefly, the medium of the treated cells was removed, then 100 µL of medium containing 0.5 mg/mL MTT was added to each well. After incubation at 37 °C with 5% CO_2 incubator for 4 h, 100 µL of 10% SDS was added and mixed to release the purple color from the cells. Last incubation was performed again in the dark for 24 h, before optical density was measured at 570 nm using ELISA reader (Sadeghi-aliabadi *et al.* 2008; Heny *et al*. 2012).

Inhibitory Effect of the Viral Titer on Infected Cell. The A549 infected SRV cell were seeded on 12 well plates with density of 10^4 cells in 2 mL medium culture/well, then incubated at 37 °C and 5% CO_2 incubator. After 24 h, medium was discarded and added with the 3 mL fresh medium containing 50 µg/mL of *A. paniculata* extract or 50 µg/mL lamivudin as positive control. After 24 h incubation, 500 µL of medium was replaced with 500 µL fresh medium containing the same concentration of extract or lamivudin. Medium replacement was done every day until 5 days incubation and the plate was kept in -20 °C until use.

RNA Extraction and PCR Analysis. Viral RNA of SRV was extracted from the infected cells using Viral RNA Extraction Kit (Qiagen,Hilden,Germany).

Briefly, 560 µL lysis buffer was pipetted into the microcentrifuge tube, and added with 140 µL cell culture media, mixed by pulse vortexing, and incubated at room temperature for 10 min. Later, 560 µL ethanol (96-100%) was added and mixed by pulse vortexing for 15 sec. After that, 630 µL of above solution was applied to the QIAamp mini column, centrifuged at 1000 g for 1 min. QIAamp mini column was placed to another collection tube, and the tube containing the filtrate was discarded. Then, the column washed twice with washing buffer and the bound RNA in the column was eluted with 60 µL of elution buffer. The viral RNA was analyzed using RT PCR. Complementary DNA of the viral RNA was synthesized by cDNA synthesis Kit (Fermentas,Vilnius, Lithuania). Briefly, the 20 µL of mix reagent containing SRV primer set consisting of an upstream primer '5-CACCTCTYTRCTYAYAGAGCTGA-3' and a downstream primer 5'-GAAACTGCGCCTGTCT-3' and dNTPs was mixed with 5 µL of RNA sample. PCR amplification was performed by following protocol: denaturation for 2 min at 92 °C, amplification for 10 cycles consisting of 10 sec at 92 °C, 30 sec at 60 °C and 2 min at 68 °C, continued with amplification for 20 cycles consisting of 15 sec at 92 °C, 30 sec at 60 °C and 2 min at 68 °C, with time prolongation of 20 sec for each cycle and ended with an extension at 68 °C temperature for 7 min (Iskandriati *et al*. 2010).

RESULTS

HPLC Analysis of the Extract. Content of active compounds andrographolide was analyzed by HPLC. The results of HPLC analysis described on the chromatogram (Figure 1) which showed that the ethanol extract of *A. paniculata* contains andrographolide.

Cytotoxicity Assay. Cytotoxic effect of *A. paniculata* extract against A549 cell was listed in Table 1, where the cytotoxic activity increased with increasing concentration of the extract treatment as indicated by decreasing of cell viability. Furthermore, based on the percent inhibition of cell proliferation

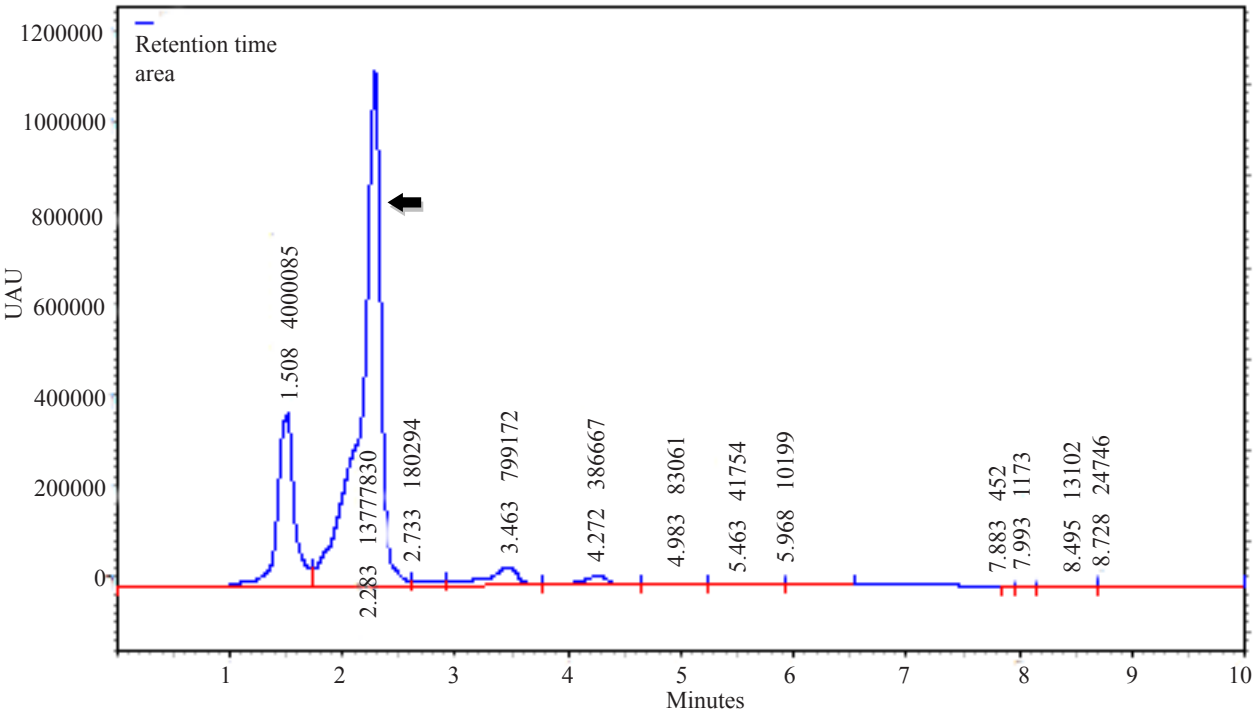

Figure 1. The chromatogram of *A. paniculata* extract on HPLC analysis. The extract was macerated using 96% ethanol, and injection volume was 1000 ppm. ⬅ Andrographolide.

Table 1. Cytotoxic activity of *A. paniculata* ethanol extract against A549 cells by MTT method

Extract concentration (µg/mL)	Cell viability (%)	Inhibition of proliferation (%)	IC50 (µg/mL)
0	100	0.00	
6.25	142	-41.66	
12.50	109	-8.98	
25	96	4.14	124.30
50	71	28.54	
100	69	30.84	
200	43	57.42	

and by probit analysis, IC_{50} was 124.3 µg/mL. This obtained IC_{50} value was large enough, suggesting that *A. paniculata* extract was not toxic to A549 cells, making it possible to provide treatment of A549 cells transfected with SRV.

Immunostimulant Activity of *A. paniculata.* *A. paniculata* ethanol extract stimulated limphocyte cell proliferation at all concentration of extract treatment from 1 to 16 µg, as shown in Figure 2, where the value of stimulation index was greater than one compared to the control non treated cell. Similarly, in Figure 3, it appears that the percentage of increasing proliferation are at the range of 27 to 38%, and the highest percentage to increase proliferation was resulted from the treatment of 1 µg/mL *A. paniculata* ethanol extract.

Antiviral Activity of *A. paniculata.* The value of cycle threshold (Ct) represents the detection of amplification product in a particular cycle on PCR analysis. Smaller initial Ct value means that more targets are amplified, whereas larger final Ct values indicate fewer targets are amplified. From Table 2 it appears that on third day after sample treatment, *A. paniculata* ethanol extract inhibited the virus titer similar to the positive control Lamivudine. Both of the ethanol extract and positive control have a Ct value greater than the negative control which indicated that *A. paniculata*, similar as Lamivudine,

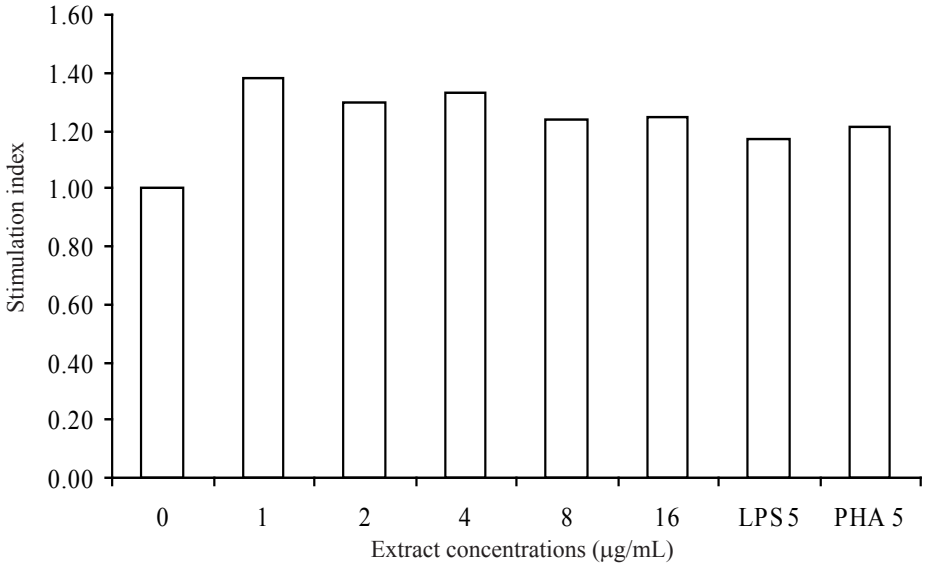

Figure 2. Lymphocyte cell proliferation stimulation index of *A. paniculata* ethanol extract on different concentration.

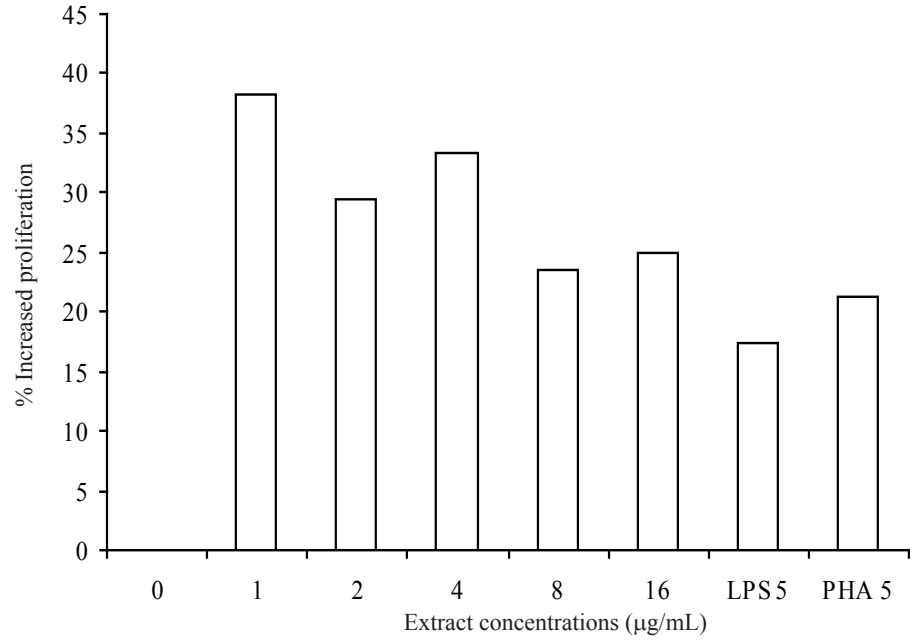

Figure 3. Percentage of increasing proliferation of lymphocyte cell after 72 h incubation on treatment with *A. paniculata* ethanol extract at different concentration.

Table 2. Cycle threshold (Ct) resulted from the Real Time PCR analysis of the medium cell culture on 3rd day after treated with *A. paniculata* ethanol extract

Extract/positive control	Ct1	Ct2	Average Ct
A. paniculata ethanol extract	18.16	17.89	18.03
Lamivudin (positive control)	18.83	18.97	18.90
Negative control	17.62	17.60	17.60

Table 3. Cycle threshold (Ct) resulted from the Real Time PCR analysis of the cell culture medium on 5th day after treated with *A. paniculata* ethanol extract

Extract/positive control	Ct1	Ct2	Average Ct
A. paniculata ethanol extract	15.30	15.52	15.41
Lamivudin (positive control)	20.04	20.04	20.04
Negative control	14.55	14.80	14.68

The negative control was cell culture medium without treatment of *A. paniculata* ethanol extract.

has antiviral activity of inhibiting virus replication. Five day after treatment, the activity of ethanol extract in suppressing virus titer was decreased, but activity of positive control Lamivudine was increased, however *A. paniculata* ethanol extract still showed higher antiviral activity than negative control (Table 3).

DISCUSSION

The results showed that the ethanol extract of *A. paniculata* used in this study were able to increase the proliferation of lymphocyte cells at low concentration (1-16 µg/mL). Other studies have also reported that *A. paniculata* extract increases the lymphocyte cell proliferation at low concentrations, for examples, *A. paniculata* dichloromethane extract significantly augments the proliferation of human peripheral blood lymphocytes (hPBL) at low concentrations (Chao & Lin 2010). Aqueous extract of *A. paniculata* also showed significant increased in cellular immunological indicators such as red blood cells, white blood cells, and thrombocytes at low concentration in Tilapia fish *Oreochromis mossambicus* (Prasad & Mukthiraj 2011). Furthermore, *in vivo* studies using animals indicated that *A. paniculata* extract exhibit a significant increase in blood lymphocyte count in both normal and type 2 diabetic rats (Radhika *et al.* 2012).

It was alleged that one of the active compounds, andrographolide contained in the extract, was responsible for the lymphocytes cell proliferation. HPLC analysis on the extract showed that the extract was indeed contained andrographolide. Previous research reported that andrographolide was able to modulate the innate immune response by regulating both classical and alternative activation of macrophages, and regulate specific antibody production as well as antigen-specific IL-4 producing splenocytes (Wang *et al.* 2010). Ethanol extract and purified diterpen andrographolide of *A. paniculata* have been shown to induce significant stimulation of antibody and also stimulate macrophage migration, and *in vitro* proliferation of splenic lymphocytes. However, it was lower with purified andrographolides than with the ethanolic extract indicating presence of substances other than andrographolide that may be contributing towards immunostimulation (Puri *et al.* 1993).

The activity of the extract in inhibiting the viral titer in A549 cells transfected with SRV showed that the 50 µg/mL extract could inhibited the viral titer equivalent to the positive control of lamivudine at the same concentration. Hereinafter, the cytotoxicity testing showed that the *A. paniculata* extract was not toxic to the A459 cell line, because it has a relatively high IC_{50} value. Previous study reported similar results where n-hexane and methanol extracts of *A. paniculata*, and seven compounds showed anti-HIV activity with 50% effective concentration (EC_{50}) of 49 and 57 µg/mL respectively (Reddy *et al.* 2005). Our results were similar to Wiart *et al.* (2005) which demonstrated andrographolide viricidal activity against herpes simplex virus 1 (HSV-1) without significant cytotoxicity. Lin *et al.* (2008) also demonstrated that the *A. paniculata* ethanol extract and andrographolide inhibit the expression of Epstein-Barr virus (EBV) lytic proteins during the viral lytic cycle in P3HR1 cells, an oral lymphoma cell line latently infected by EBV. Andrographolide inhibits the production of mature viral particles and was not toxic to P3HR1 cells.

In conclusion, our result indicated that *A. paniculata* ethanol extract inhibited the SRV replication almost similar to the positive control Lamivudine, and the extract also stimulated lymphocyte cell proliferation at low concentration.

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